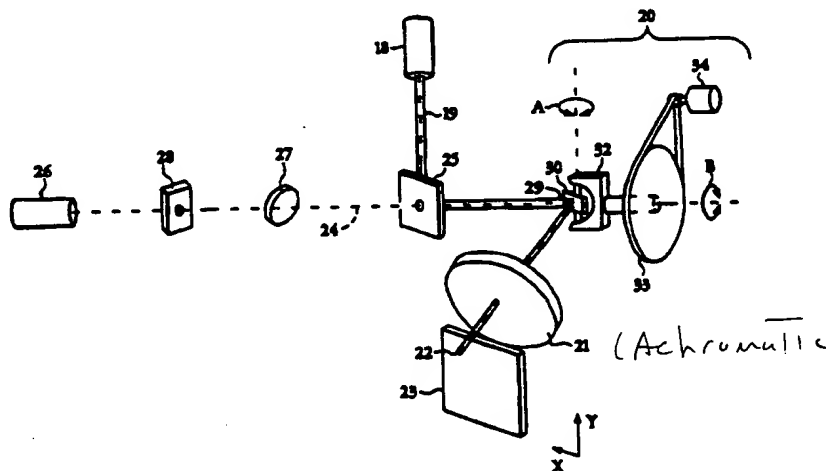




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(21) International Application Number: PCT/US95/13531 (22) International Filing Date: 20 October 1995 (20.10.95) (30) Priority Data: 351,603 8 December 1994 (08.12.94) US (71) Applicant: MOLECULAR DYNAMICS, INC. [US/US]; 928 East Arques Avenue, Sunnyvale, CA 94086 (US). (72) Inventor: KAIN, Robert, C.; 1411 San Thomas Aquino Road, San Jose, CA 95130 (US). (74) Agents: SCHNECK, Thomas et al.; Schneck & McHugh, P.O. Box 2-E, San Jose, CA 95109-0005 (US).	(81) Designated States: AU, CA, FI, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE). Published <i>With international search report.</i>	

(54) Title: FLUORESCENCE IMAGING SYSTEM EMPLOYING A MACRO SCANNING OBJECTIVE



(57) Abstract

Fluorescence imaging system includes objective (21) which is achromatic and has an external entrance pupil (29), serves as a condenser for the system, and positioned above sample (23) in close proximity to one another. Laser (18) directs collimated light (19) to scan device (20) located at entrance pupil (29). Scan device (20) reflects, refracts, or diffracts light through objective (21) to illuminate a spot (22) on the sample's surface, and illuminates a line or an area on the sample surface by varying the angle of laser light into objective (21). Sample (23) emits fluorescent light (24) in response to the illumination. The fluorescent light (24) is collected by objective (21) and passes through the system along the path of the illumination light. Wavelength-discriminating dichroic filter (25), placed along the optical axis between laser (18) and objective (21), directs fluorescent light (24) onto photodetector (26) to produce a signal representing the sample surface emitting the fluorescent light. Display (37) displays the digitized data in a raster format.

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Description

Fluorescence Imaging System
Employing a Macro Scanning Objective

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Technical Field

The present invention relates to laser scanning
~~imaging systems, particularly for use in fluorescence~~
imaging.

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Background Art

Fluorescence microscopy is often used in the
fields of molecular biology, biochemistry and other life
sciences. One such use is in identifying a specific
15 antigen using antibodies. Antibodies are proteins pro-
duced by vertebrates as a defense against infection.
They are made of millions of different forms, each has a
different binding site and specifically recognizes the
antigen that induces its production. To identify an
20 antigen, a sample of cells is provided that contains
specific antibodies coupled to a fluorescent dye. The
cells are then assessed for their fluorescence. Taking
advantage of the precise antigen specificity of anti-
bodies, the cells having fluorescent properties are known
25 to contain a specific antigen.

Originally, the fluorescence of cells was
assessed manually by visual inspection, using conven-
tional microscopy. This proved time-consuming and
costly. The need for high-speed automated systems became
30 manifest. Many high-speed imaging systems, such as
confocal microscopes, are available for assaying cell
samples. The illumination and collection optics, along
with their relative geometry, determine in large part the
parameters of the other system elements.

35 A prior art high-speed imaging system is shown
in Fig. 1 and includes an F-theta objective 10 positioned
above a sample 11 so that the surfaces of the objective
are perpendicular to the sample's normal. A laser light

source 12 produces a beam 13. The objective 10 directs the beam 13 to illuminate a spot on the sample's surface. An oscillating reflective surface 14 is disposed at the pupil 15 of the system, between the light source 12 and the objective 10, to deflect the beam 13 back and forth along one axis. The sample is placed on a table to move the sample in a direction perpendicular to the first scan direction, thereby resulting in a two dimensional scan pattern on the sample's surface. The objective is not designed for coaxial collection resulting in light reflected from the sample surface being collected by a condenser assembly 16 that is separate and apart from the objective. Such a geometry results in increased system footprint, increased optical complexity, and a limitation of solid angle collection. The collected light is then imaged on a photo-detector 17. The design of a classical F-theta lens is primarily for monochromatic illumination. As a result, such lenses lack good polychromatic performance. Therefore, the objective 10 manifests lateral and axial chromatic aberrations over a broad band of wavelengths.

A prior art high-speed imaging system, similar to that described with respect to Fig. 1, is disclosed by Richard L. Shoemaker et al., "An Ultrafast Laser Scanner Microscope for Digital Imaging Analysis", IEEE Transactions on Biomedical Engineering, Vol. BME-29, No. 2, February 1982, pp. 82-91. The principal difference between these two systems concerns the scanning device. Instead of a galvanometric scanner, Shoemaker et al. require the use of a rotating polygon mirror to scan the spot over the sample's surface.

Another prior art high-speed imaging system is disclosed in U.S. Pat. No. 4,284,897 to Sawamura et al. in which laser light is reflected through two galvanometric mirrors and one dichroic mirror to direct a beam through an objective and illuminate a spot on a sample's surface. The galvanometric mirrors are swung in appropriate directions to allow the spot to scan over the

entire surface of the sample. In response to the illuminating spot, the sample emits fluorescence light. The objective, serving as a condenser lens, transmits the light back through a first dichroic mirror. Positioned
5 behind the first dichroic mirror is a second dichroic mirror that splits the fluorescent light into a light produced by a cytoplasm and light produced by a nucleus. ~~The cytoplasm fluorescence and the nucleus fluorescence~~ is transmitted to a respective photo-detector.

10 A disadvantage of the prior art systems is that in addition to the illumination optics, additional optics are required either to scan the beam on a sample or to collect light emitted from a macro size sample, thereby increasing the systems' cost and size.

15 It is an object, therefore, of the present invention to provide a high-speed, low cost, laser scanning system that will provide point by point fluorescent imaging of a sample on a macro scale.

20 It is a further object of the present invention to provide an fluorescence imaging system of a substantially smaller size than the prior art systems that affords a larger scan field than existing coaxial illumination and collection systems.

25 Disclosure of the Invention

These objectives have been achieved by positioning a telecentric lens, that has an external pupil and is achromatic, above a sample so that they are both in close proximity with one another. The lens forms the
30 objective of the system. The objective is defined as the system's lens that is closest to the sample and which has as its front focus, the sample. A laser produces a collimated beam of coherent light that is directed through the objective to illuminate a spot on the sample's
35 surface, thereby stimulating a small region of the sample to emit fluorescent light. The spot usually has a diameter close to the limit defined by the laws of diffraction. The objective also serves as a condenser

and collects the fluorescent light emitted by the sample. The objective directs the collected light back along the identical path traveled by the incident beam, but in the opposite direction. A wavelength-discriminating dichroic filter is placed along the optical axis between the laser and the objective to separate the fluorescent light from the incident beam and direct the fluorescent light onto a photo-detector to produce a signal representing the sample surface emitting the fluorescent light. To obtain a full field view of the sample, a two dimensional scanning device, with a reflecting element having a scan center positioned at the pupil of the system, scans the spot over the entire surface of the sample. A display device is provided and synchronized with the scanning device to reproduce an image of the sample.

Brief Description of the Drawings

Fig. 1 is a simplified side view of a laser scanning microscope of the prior art.

Fig. 2 is a perspective view of optical components of the present invention.

Fig. 3 is a detailed view of a scanning beam passing through the objective shown in Fig. 2.

Fig. 4 is a simplified side view of the optical components shown on Fig. 2 including a video display system to reproduce an image of the sample in accordance with the present invention.

Best Mode for Carrying Out the Invention

Fig. 2 shows a laser 18 producing an incident beam 19 of light. It is preferred that the laser produce a collimated beam of coherent light. However, it is possible to use a non-coherent light source optically coupled to collimating optics to create an incident light beam, e.g., a light emitting diode.

The beam 19 is reflected on a two dimensional scanning device 20 and directed through an achromatic objective 21, which also serves as a condenser. One

advantage of having collimated light entering the objective is that it renders the system less sensitive to changing objectives. The objective 21 directs the beam 19 to illuminate a spot 22 on a sample 23, thereby stimulating a small region of the sample to emit fluorescent light. The fluorescent light is collected by the objective 21 and directed, as a retro-beam 24, back along the identical path of the incident beam 19, but in an opposite direction. A dichroic filter 25 separates fluorescent light from the incident beam and images the retro-beam 24 onto a photodetector 26. The dichroic filter 25 could be employed so that it transmits laser light and reflects fluorescent light. Alternatively, the laser light could be reflected and the fluorescent light transmitted so that the fluorescent light impinges on the photodetector 26, which is the preferred embodiment shown in Fig. 2. It is to be understood that any type of beamsplitter may be employed, so long as it is capable of separating the incident beam from the fluorescent light. For example, a polarization sensitive beamsplitter may be used to achieve the separation. This embodiment could include a 1/4 waveplate positioned between the beamsplitter and the objective. This would cause the incident beam exiting the 1/4 waveplate to be circularly polarized. Also, additional focusing optics 27 and a back aperture 28 may be present to further shape the retro-beam 24 as desired. The sample 23 is illuminated point by point by scanning the spot 22 in a raster scan fashion over the entire surface of the sample 23, to obtain a full-field image of it.

Referring to Fig. 3, the objective 21 affords coaxial illumination and collection. With respect to the incident beam 19, the objective 21 is afocal in the image plane. It is preferred that the objective is telecentric. The telecentricity of the objective results in the sample 23's surface always lying at a right angle with respect to the incident beam 19, exiting the objective 21. With respect to the incident beam 19, the objective

21 1/
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input &
output
len!

plane is proximate to the sample 23. The beam 19 is shown entering the objective 21 at three different positions, with the incident beam 19 having a different angle of incidence at each position. Regardless of the incident beam 19's angle of incidence on the objective 21, the incident beam 19 exiting the objective 21 is orthogonal to the sample 23's surface. One advantage of having this telecentric objective is that it renders the system magnification relatively insensitive to errors in focus position. In addition, the achromaticity of the objective 21 allows it to operate over a broad band of wavelengths of light, e.g., the primary wavelength plus approximately 200 nm, or greater, while maintaining axial and lateral aberrations below the effective resolution of the system. This allows the objective 21 to operate with lasers of various wavelengths and to collect light from a wide variety of fluorochromes.

The specifications of two implementations of the objective are as follows:

20	Primary Wavelength (nm)	488	488
	Focal Length (mm)	30	76
	Scan Angle (degrees)	+/- 3	20
	Scan Length (mm)	3	52
25	Telecentricity (degrees)	< 1	< 1
	F/Number (@ 488 nm)	f/1.5	f/4.8
	Resolution (microns)	= 1.5	= 5 measured at (1/e ²)
	Working Distance (mm)	= 3	20
30	Collection Wavelength (nm)	488-650	488-650
	Collection F/Number	f/1.5	-f/2 to f/2.5

It is important to note that the specifications listed above are merely exemplary of the lenses used in the present invention. The parameters may be varied as needed to adapt a system to a particular operation.

Referring again to Fig. 2, an important feature of the objective 21 is that it defines an external pupil

29 of the system, which is positioned at the scan center. Any scanning mechanism that provides a two dimensional scan may be used, e.g., a rotating polygonal mirror, rotating holographic scanner, or oscillating prisms.

5 Also, an acousto-optic deflector or a penta-prism scanning deflector may be employed. The preferred embodiment, however, is to employ a scanning system having one beam reflecting element in the path of the incident beam which is pivotable about two perpendicular axes. The
10 reflecting element is a planar mirror 30, but this is not essential as refractive or diffractive deflecting elements may also be used. The mirror is supported on spindles 31 by a forked bracket 32 and, therefore, pivotable about axis A. The mirror 30 may be moved by
15 any means known in the art, but is typically a galvanometer mirror. The bracket 32 is attached at one end to wheel 33 that is driven by a stepper motor 34. The motor 34 drives the wheel 33 to pivot the mirror 30 about axis B.

20 Referring to Fig. 4, the retro-beam 24 is shown emanating from a plurality of points on the sample 23's surface. The retro-beam 24 is imaged on a light detector 26. Although any light detector may be used, it is preferred to use a photomultiplier tube. The signal from
25 the photomultiplier tube passes through electrical connections 35 to a signal processor 36 of a video display system including a video display screen 37. The signal from the photomultiplier tube 26 modulates the intensity of the image signal transmitted from the processor 36
30 through the output line 38 to the display screen 37. A scanning signal generator 39 supplies electrical signals to the scanning apparatus 20 through electrical connections 40. The scanning apparatus moves in response to the generator 39's signals. The signal from the photo-
35 multiplier tube is digitized and stored in memory and can be simultaneously scanned onto a display.

An obvious extension of the invention is in the area of reflection imaging. That is, the reflected laser

beam could be collected at the detector instead of the
fluorescent beam. Both the reflected beam and the
fluorescent beam could be read at the detectors if a
second dichroic beamsplitter was positioned after the
5 primary dichroic beamsplitter. Or in a like manner,
multiple fluorescent labels could be detected by using
multiple secondary beamsplitters and detectors.

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Claims

1. An optical scanning system comprising,
an objective disposed proximate to a sample,
5 defining an optical axis, said objective positioned to
receive an incident beam of light and directing said beam
onto the sample, producing an illuminated spot thereon
~~with said objective collecting light emitted from said~~
spot and forming a retro-beam, said objective having an
10 external entrance pupil sufficiently distant from the
objective to be positioned proximate to a scanning
device,
a beam source for emitting said incident beam,
means, in said optical axis, for scanning said
15 spot, on said sample surface, and
means positioned in said optical axis between
said beam source and said objective for separating said
incident beam from said retro-beam, wherein said
objective directs said retro-beam onto said separating
20 means with said separating means directing said
retro-beam onto a detector.
2. The optical scanning system of claim 1 wherein said
25 objective is afocal at the entrance pupil.
3. The optical scanning system of claim 1 wherein said
objective is telecentric.
30
4. The optical scanning system of claim 1 wherein said
separating means is a dichroic filter.
- 35
5. The optical scanning system of claim 1 wherein said
separating means is a first reflector.

6. The optical scanning system of claim 1 further including means, positioned between said beam source and said separating means, for changing the diameter of said incident beam.

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7. The optical scanning system of claim 1 wherein said beam source is a non-coherent source of light optically coupled to collimating optics.

10

8. The optical scanning system of claim 1 wherein said beam source comprises a light emitting diode optically coupled to collimating optics.

15

9. The optical scanning system of claim 1 wherein said separating means is a mirror having a diameter greater than the diameter of said incident beam and smaller than the diameter of said retro-beam, with the diameter of the retro-beam being substantially larger than the diameter of the incident beam.

20

10. The optical scanning system of claim 1 wherein said scanning means includes a concave mirror positioned at the pupil of the system.

25

11. The optical scanning system of claim 1 wherein said scanning means includes a convex mirror positioned at the pupil of the system.

30

12. The optical scanning system of claim 1 wherein said scanning means includes a refractive scanning deflector positioned at the lens pupil.

35

-11-

13. The optical scanning system of claim 1 wherein said scanning means includes a diffractive scanning deflector positioned at the pupil of the system.

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14. The optical scanning system of claim 1 wherein the scanning means includes a rotating penta-prism scanning deflector positioned at the pupil of the system.

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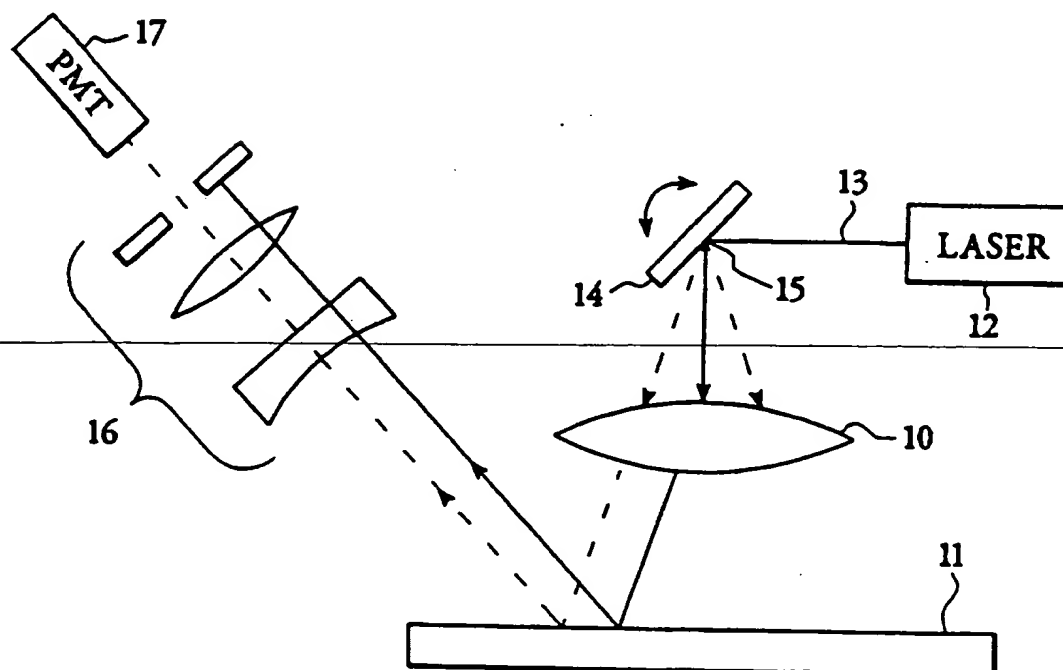
$\frac{1}{3}$ 

FIG. 1 (PRIOR ART)

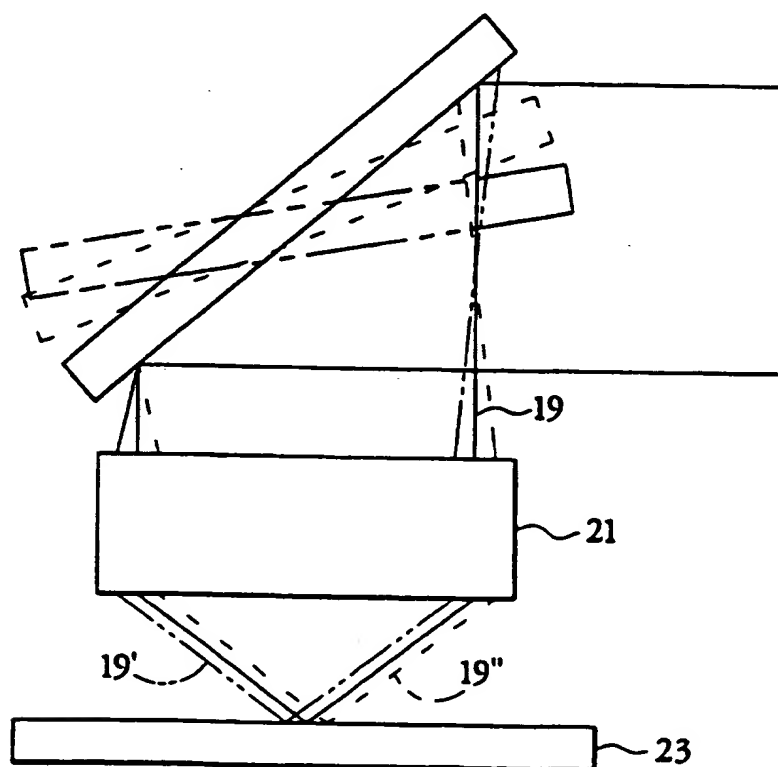


FIG. 3

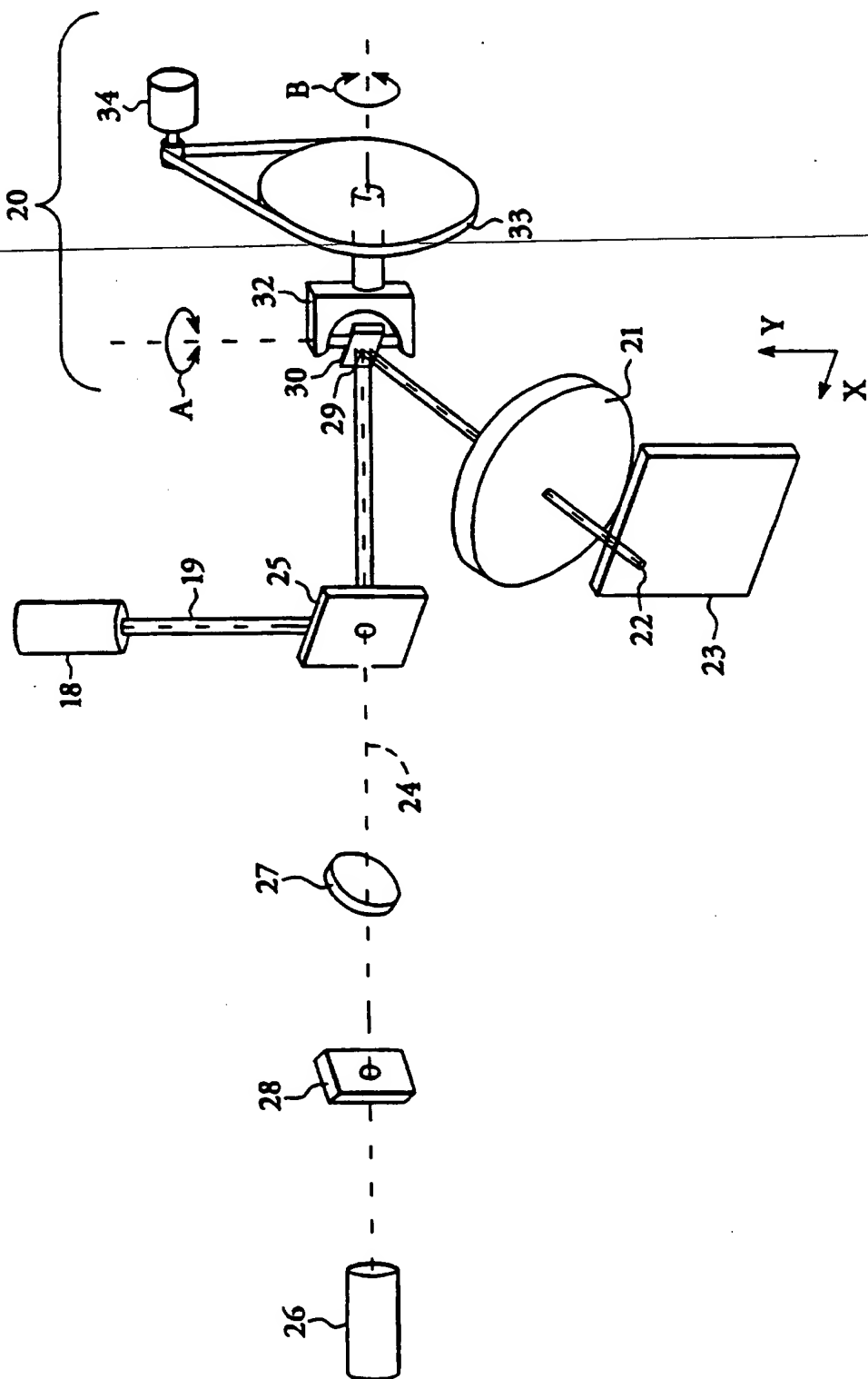


FIG. 2

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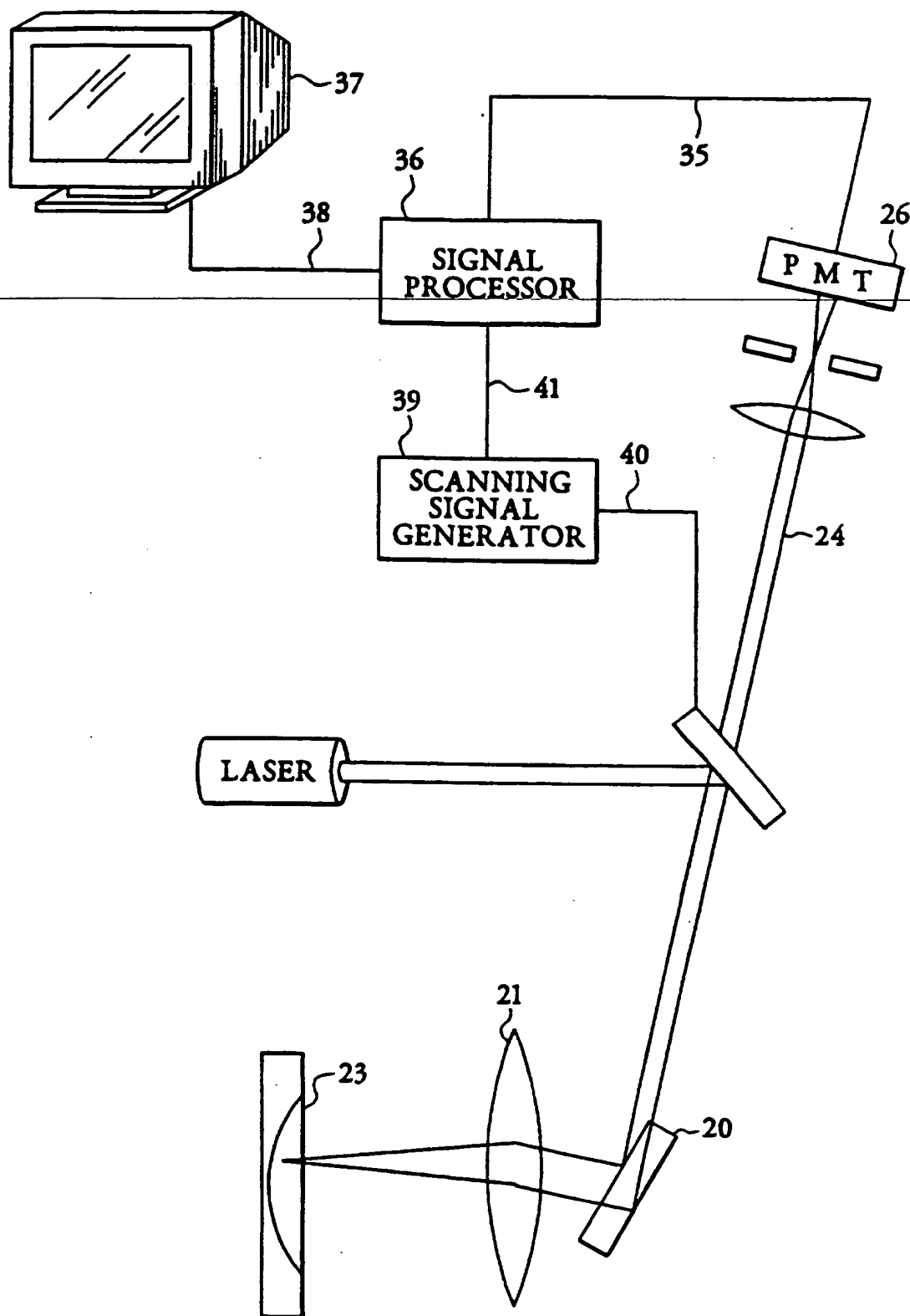


FIG. 4

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US95/13531

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :H01J 03/14; G01N 21/64

US CL :250/234-236, 461.1, 461.2, 458.1; 356/30

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 250/234-236, 461.1, 461.2, 458.1; 356/30

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS

S ACHROMATIC(3A)OBJECTIVE AND EXTERNAL(3A)PUPIL AND SCAN?
S L1 AND FLUORESC?**C. DOCUMENTS CONSIDERED TO BE RELEVANT**

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A, E	US, A, 5,260,578 (BLITON ET AL) 09 NOVEMBER 1995, SEE ENTIRE DOCUMENT	1-18
A	US, A, 4,058,732 (WIEDER) 15 NOVEMBER 1977, SEE ENTIRE DOCUMENT	1-25
A	US, A, 3,657,537 (WHEELLESS, JR. ET AL) 18 APRIL 1972 SEE ENTIRE DOCUMENT	1-12

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

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